CHEMISTRY, ECOLOGY AND BIOLOGICAL ACTIVITY OF THE HAPLOSCLERID SPONGE *OCEANAPIA* SP.; CAN ECOLOGICAL OBSERVATIONS AND EXPERIMENTS GIVE A FIRST CLUE ABOUT PHARMACOLOGICAL ACTIVITY?

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A new species of *Oceanapia* was discovered around Moen Island, Chuuk Lagoon, Micronesia, during collections made in 1994-1995. The appearance and growth form closely resembled that of *Oceanapia sagittaria*, which is widely distributed throughout the Western Pacific. Unlike the Chuuk species, however, *O. sagittaria* has microscleres (sigmas and toxas) and thicker oxeas, suggesting to us it may be new. We examined whether this conspicuous, red sponge was chemically defended against generalist and more specialized fish predators. Methanol extracts of the sponge were highly deterrent in field feeding assays against generalist reef fish at physiological concentrations. This extract also deterred feeding by the spongivorous angelfish, *Pomacanthus imperator*, in laboratory feeding experiments at the same concentration. Based on our field observations and fish feeding experiments, different pharmacological screens were undertaken to demonstrate possible targets of these compounds. Kuanoniamine C and D showed insecticidal activity against the polyphagous larvae of *Spodoptera littoralis*, and toxic effects against the brine shrimp *Artemia salina*; N-deacyl derivative did not show a pronounced activity; whereas cytotoxicity screens against HELA and MONO-MAC-6 tumor cells found that all three compounds revealed considerable cytotoxicity. ☐ *Porifera, Oceanapia, secondary metabolites, predation, feeding deterrency, Micronesia, kuanoniamines.*

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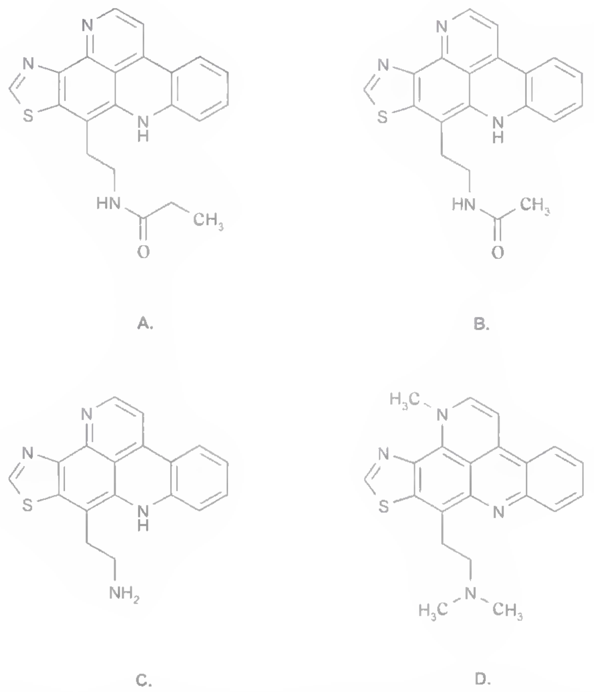
Despite considerable progress achieved in modern synthetic chemistry, natural products are still an indispensable source for the development of new pharmaceuticals and plant protectants. The frequent occurrence of bioactive compounds in nature is due to selective forces (e.g. predation or attack by pathogens), which have shaped and optimized a highly efficient chemical defense armory in many plants, animals and microorganisms (Harborne, 1993). For decades scientists have relied on ethno-pharmacological knowledge to obtain initial indication on the biological and pharmacological properties of terrestrial plants. In contrast, there is almost no equivalent information available for the marine environment, requiring the implementation of other strategies to achieve fast and environmentally sensible bioprospecting.

Marine organisms are a rich source of novel, often unusual, secondary metabolites (Faulkner, 1996). Researchers often focus only on the ecology of secondary metabolites from marine organisms (Pawlik, 1993; Hay, 1996; Wink, 1998), or on the chemistry and pharmacological activity of the new compounds (Eder et al., 1998; Faulkner, 1996; Eder et al., 1999). Ecological studies focus mostly on assumed defensive functions of secondary metabolites, such as predator deterrence (Paul, 1992; Pawlik et al., 1995), prevention of fouling (De Nys et al., 1991), or inhibition of overgrowth (Thacker et al., 1998). Chemical studies report mainly on the structure elucidation of new compounds and their pharmacological activities, such as cytotoxicity (Steube et al., 1998) and anti-microbial activity (Edrada et al., 1996).

Paul (1988) suggested that ecological experiments could be useful as a first indication for the presence of pharmacological active compounds, although at that time there was no clear empirical support for this idea. In this study, we show that ecological observations and



A. B. C. D.

FIG. 1. Major metabolites isolated from *Oceanapia* sp. A, kuanoniamine C; B, kuanoniamine D; C, N-acetyl-kuanoniamine D; D, dercitin.

experiments can indeed be used as initial indicators of possible, potential pharmacological properties of the compounds involved (Schupp et al., 1999a, 1999b).

MATERIALS AND METHODS

COLLECTION AND ISOLATION. *Oceanapia* sp. was collected at 1-3m depth on reef flats near the southern tip of Moen Island, Chuuk Lagoon, Federated States of Micronesia. Immediately after digging the basal 'root' of the sponge from the substrate, samples were separated *in situ* into the translucent capitum, fistule and base. After separation the different sample components were frozen and stored at - 20°C, until subsequent freeze-drying and extraction in 100% methanol.

CHEMISTRY. The crude methanol extract was evaporated under reduced pressure and chromatographed on a silica gel column (elution with $CH_2Cl_2/MeOH/NH_4OH$, 70:30:3). The pyridoacridine alkaloids kuanoniamine C (Fig. 1A), kuanoniamine D (Fig. 1B), and N-deacetyl-kuanoniamine D (Fig. 1C) were obtained after a final purification on a RP-18 silica gel column ($MeOH/H_2O/TFA$, 70:30:0.5)(Eder et al., 1998).

The N-deacyl derivative could only be isolated from the sponge in trace amounts. Therefore, we synthesized the compound through acid hydrolysis of kuanoniamine C, which provided a sufficient quantity of the compound for subsequent bioassays. Kuanoniamine C (65mg) was dissolved in 120mL MeOH and the same volume of 2N HCl was added. The reaction mixture was stirred for 48hrs at 85°C under reflux. The sample was dried and the resulting red solid was purified by RP-18 column chromatography, using the solvent system described above, to yield 49mg of the new compound. Structure elucidation was accomplished by NMR and mass spectrometry (Eder et al., 1998).

BIOLOGICAL ACTIVITY. *Experiments with insects.* Neonate larvae of the vigorous pest insect, *Spodoptera littoralis*, were used to test if the isolated compounds had any insecticidal properties. This assay allowed determination whether compounds were toxic towards insect larvae, or if they reduced or inhibited larval growth. It was not possible to determine whether reduced growth was due to inhibition of physiological processes and metabolism, or reduced food consumption caused by feeding deterrence. Larvae of *S. littoralis* were obtained from a laboratory colony, reared on an artificial diet under controlled conditions, as described by Srivastava & Proksch (1991). Feeding studies were conducted with neonate larvae (n=20) maintained on an artificial diet which had been treated with various concentrations (13-373ppm) of the compounds under study. *Spodoptera littoralis* was offered the spiked diet over a range of 5-6 concentrations in a chronic feeding experiment. After 6 days, surviving larvae were counted, weighed, and compared to controls. LC_{50}s were calculated from the dose-response curves by probit analysis (Eder et al., 1998).

Brine Shrimp Assay. This assay was used to determine if the isolated compounds were toxic towards small marine organisms, although no information was forthcoming on the mode of action, or on how specific the toxicity was directed towards the test organisms (*Artemia salina*). Eggs of this species were kept for 48hrs in artificial seawater, as described previously (Meyer et al., 1982), and the nauplii (n=20) were introduced into vessels with brine, containing various concentrations (5-100µg/mL) of the compounds under test (each concentration in triplicate). DMSO (10µL/mL brine) was added to improve solubility. After 24hrs the surviving larvae were counted and compared to controls. LC_{50}s were calculated from the dose-response curves by probit analysis (Eder et al., 1998).

Cytotoxicity Studies. Information on the selectivity and possible intracellular targets were tested by analysing the effects of kuanoniamine C and D, and the new compound N-deacetyl-kuanoniamine D, on cell growth and differentiation of two different human cell lines. These cell lines, MONO-MAC 6 and HELA, were deposited in the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). Cultures were mycoplasma-free, and were cultivated under standardized conditions (Drexler et al., 1995). For all experiments, exponentially-growing cells were used, with a viability exceeding 90%, as determined by trypan blue staining. The final concentration used in experiments was 10^5cells/mL. Experiments were conducted using 100IU/mL penicillin G and 100mg/mL streptomycin. Concentrated stock solutions of the test compounds were prepared in ethylene glycol monomethyl ether, and stored at -20°C. For the cytotoxicity analysis, cells were harvested, washed, and resuspended in a final concentration of 10^5cells/mL. These solutions were seeded in triplicate, in 90µL volumes, in 96-well flat bottom culture plates (Nunc). Test compounds in 10µL, obtained by diluting the stock solution with a suitable quantity of growth medium, were added to each well (Eder et al., 1998). Cultures were incubated for 48hrs at 37°C in a humidified incubator with 5% CO_2. Cytotoxicity was determined by incorporation of $[^3H]$-thymidine (Steube et al., 1992). Radioactive-incorporation was carried out for the last 3hrs of the 48hr incubation period. One µCi of [methyl-^{3}H]-thymidine (Amersham-Buchler, Braunschweig, Germany; specific activity 0.25mCi/µmol), was added in 20µL volumes to each well. Cells were harvested on glass fiber filters with a multiple automatic sample harvester, and radioactivity was determined in a liquid scintillation counter (1209 Rackbeta, LKB, Freiburg, Germany). Media with 0.2% ethylene glycol monomethyl ether were included in the experiments as controls (Eder et al., 1998).

ECOLOGY. *Distribution of secondary metabolites.* Extract and secondary metabolite concentrations were determined for the basal, fistular and capitum subsamples of *Oceanapia* sp., to see whether there were any differences in compound concentrations between these subsamples of the sponge, correlated with differences in exposure of these sponge structures (e.g. burrowing versus exposed). This strategy was essential to test the extract and sponge compounds at ecological relevant concentrations. These freeze-dried sponge subsamples were weighed before extraction, and the extract weights were also determined after removing all solvents with a rotary evaporator. Concentrations of kuanoniamine C and D in the different subsamples of the sponge were determined by HPLC quantification, following methods described by Schupp et al. (1999b).

Field feeding assays. Field feeding experiments using natural fish assemblages were conducted as an initial test for biological activity of the crude extract. We used reef fishes to determine whether the crude extract had antifeedant properties. The methanol fraction was incorporated in an artificial diet at a concentration of 7.4 % of dry mass, which represents the methanol extract concentration in the basal part of the sponge. We also tested the three major metabolites (Fig. 1A-C) at their respective fistule concentrations, against several fish species in the field at Western Shoals, Guam. Kuanoniamine C and D and the N-deacyl derivative were tested at fistule concentrations since this was considered to be the most easily accessible structure of the sponge susceptible to fish feeding (Schupp et al., 1999b).

The food was prepared according to Schupp et al. (1999b). Four food cubes, with or without one of the metabolites, were attached to a polypropylene rope by a safety pin. Ropes were placed on the reef in pairs of one treated (with one metabolite), and one control (without the metabolite) rope, and attached to coral heads at 3-5m depth. Several pairs of ropes (replicates) were set at the same time. Ropes were removed when approximately half the cubes were completely eaten. These assays were scored as the number of cubes completely eaten, and the results analyzed with a Wilcoxon signed-rank test for paired comparison (two-tailed; Schupp et al., 1999b).

Laboratory feeding assays. The angelfish, *Pomacanthus imperator*, was used as an experimental subject to determine whether or not there were differences in susceptibility between generalist and specialist predators (Thacker et al.,

TABLE 1. *In vitro* cytotoxicity of the kuanoniamine derivatives (IC_{50} values, μg/mL) to HELA and MONO-MAC-6 tumor cells in the [^{3}H]-thymidine incorporation assay (MTT), (mean ± SD, n=3; Eder et al., 1998).

Cell line	Compound		
	1	2	3
HELA	5.1 ± 1.3	1.4 ± 0.7	1.2 ± 0.2
MONO-MAC-6	1.2 ± 0.4	0.8 ± 0.1	2.0 ± 0.5

1998). Experimental food was prepared following methods described by Schupp et al. (1999b),resulting in strips of window screens that had one rectangle of treated food and one rectangle of control food (2.5x2.0cm) embedded in the screens. To determine the amount of control and treated food eaten by angelfish, the squares in the window screen served as a grid, and the number of squares where the food had been completely removed were counted (Hay et al., 1998). These results were analyzed with a paired t-test (Schupp et al., 1999b).

RESULTS AND DISCUSSION

TAXONOMY. While diving on reef flats, in sandy areas and coral rubble, a bright-red, short stocked capitum was observed protruding from the sand. The capitum was found sitting on top of a long fistule, buried in the sand, and attached to the turnip-shaped base of the sponge burrowed in the substrate (Fig. 2). Sometimes this basal portion can be buried up to 20cm deep into the substrate. The capitum is easily broken off by stronger water movements, found 'rolling' on the substrate. The capita are between 0.5-1.5cm diameter. The hollow fistules vary in length from 6-12cm and up to 1.3cm diameter. Fistules are attached to an irregular turnip-shaped main body (base), up to 10cm high, 6cm diameter, and sometimes several fistules are attached to the base of the sponge. In some cases one fistule branches into several smaller ones, protruding from the sand. The burrowing basal portion often grows around large pieces of coral rubble, or completely incorporates smaller pieces of rubble, shells, or sand into the base. The color of the sponge ranges from bright- to dull-red. The surface of the sponge is smooth, with a distinct skin-like ectosomal layer, and a very crumbly, fragile choanosome, with an abundance of foreign material. Spicules are exclusively curved oxeas, with pointed or blunt tips, ranging in size from 260-320x3-5μm. This material probably belongs to an undescribed species of *Oceanapia* (M. Kelly, pers. comm.), subsequently verified by Dr. Rob W.M. van Soest (Eder et al., 1998), and a voucher of the sponge is deposited in the Zoölogisch Museum, Amsterdam (registration number ZMA POR 11007).

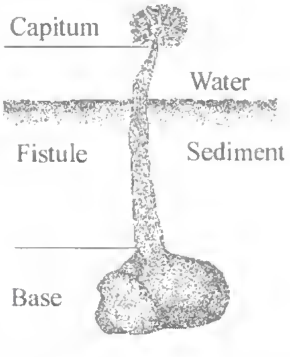


FIG. 2. Schematic drawing of *Oceanapia* sp. (Redrawn from Hooper et al., 1993).

CHEMISTRY. Three pyridoacridine alkaloids were isolated from *Oceanapia* sp. (Eder et al., 1998): kuanoniamine C (Fig. 1A), kuanoniamine D (Fig. 1B), and N-deacetylkuanoniamine D (Fig. 1C), of which kuanoniamine C and D have been previously described from an unidentified tunicate and its prosobranch mollusc predator, *Chelynotus semperi* (Carroll & Scheuer, 1990). Additionally, kuanoniamine C has also been isolated from a deep water sponge of the genus *Stelletta* (Gunawardana et al., 1989, 1992), and from *Oceanapia sagittaria* (Salomon & Faulkner, 1996). The fact that these alkaloids occur in different species, comprising more than one major phylum, gives support to the speculation that marine microorganisms could possibly be the true source of these compounds (Molinski, 1993). Conversely, there is some evidence that sponge cells are the actual producers of the alkaloids (Faulkner et al., 1999, this volume).

BIOLOGICAL ACTIVITY. The insecticidal activity of the three metabolites (Fig. 1A-C) was determined by assessing survival and growth rates after six days of exposure. From the dose-response curves obtained, LC_{50} values of 156ppm (±0.46 S.E.) for kuanoniamine C, and

TABLE 2. Results of the fish feeding experiments testing the deterrent effect of the extract and the kuanoniamine derivatives against an array of reef fish at Western Shoals, Guam.

Sponge part (compound/ extract concentration, % of dry mass)	Mean number of cubes eaten STD		N	P
	Control food	Treated food		
Methanolic extract from base , 7.4 %	3.1 ± 0.2	0.7 ± 0.7	17	<0.001
Kuanoniamine C from fistule, 1.2 %	3.5 ± 0.5	0.5 ± 0.6	19	<0.001
Kuanoniamine D from fistule, 0.4 %	3.6 ± 0.5	1.1 ± 1.2	19	<0.001
N-deacetylkuanoniamine D from fistule, 0.04 %	2.8 ± 0.7	2.5 ± 1.1	20	=0.27

59ppm (±0.30) for kuanoniamine D, were calculated by probit analysis. The new compound N-deacetylkuanoniamine D (Fig. 1C), was tested up to a concentration of 934ppm, although, in contrast to the other two compounds, no significant insecticidal activity was observed. Only the growth of larvae was reduced. This can be expressed by an ED_{50} value of 141ppm (±0.13) for the new compound (Fig. 1C).

General cytotoxicity was assessed by testing the three alkaloids (Fig. 1A-C), in the brine shrimp assay. After 24hrs of exposure, the number of surviving nauplii was counted. LC_{50} values calculated from the dose-response curves by probit analysis were 37µg/mL (±17) for one compound (Fig. 1A), and 19µg/mL (±4) for the second compound (Fig. 1B). The third compound (Fig. 1C), did not show any toxic effect up to 100µg/mL. No LC_{50} of this alkaloid was obtained due to its limited solubility at concentrations greater than 100µg/mL.

Several pyridoacridine alkaloids have been reported to exhibit significant cytotoxicity towards murine and human tumor cell lines (Molinski, 1993). In this study, we analyzed the effects of the three compounds (Figs 1A-C), on cell growth towards two different human cell lines using [^{3}H]-thymidine incorporation. This cytotoxicity assay is used to determine the capability of cells to synthesize DNA during the cell cycle. The validity of the method applied in this study has been documented previously (Arnould et al., 1990). Table 1 summarizes the results obtained with the two cell lines. The small amount of ethylene glycol monomethyl ether (0.1%), used to solubilize the compounds, did not affect growth of the tumor cells. Each alkaloid was tested for its cytotoxic activity at a range of concentrations (0.1-20µg/mL).

Suppression of [^{3}H]-thymidine incorporation into cells treated with the three compounds (Fig. 1A-C), was observed for each of the two cell lines.

Intercalation with DNA has been demonstrated previously for dercitin (Fig. 1D) (Gunawardana et al., 1988; Burres et al., 1989), a marine natural product closely related to the kuanoniamines. Based on obvious structural similarities of these three alkaloids (Fig. 1A-C) with dercitin, it may be hypothesized that the kuanoniamines also interact with DNA by intercalation.

In addition to our own testing, kuanoniamine C was tested with the *in vitro* disease-oriented primary antitumor screen on a panel of 60 cell lines at the National Cancer Institute (NCI), in Bethesda, Maryland, U.S.A.

The *in vitro* assays showed that most cell lines were fairly sensitive against kuanoniamine C at concentrations, ranging from 4.93 x 10^{-05} molar to 3.11 x 10^{-08} molar for the GI_{50}, and a concentration of 1.86 x 10^{-06} molar as the mean graph midpoint (mean concentration required over all cell lines; Monks et al., 1991). The GI_{50} is an interpolated value representing the concentration at which the percentage growth of exposed cells used in the assay is 50% compared to that of non-exposed cells (Monks et al., 1991). One of the breast cancer cell lines (MCF7), was extremely sensitive against kuanoniamine C, with a concentration of 3.11 x 10^{-08} molar for the GI_{50}.

Based on the successful performance of the *in vitro* screen, NCI subsequently requested further material for *in vivo* studies.

The cytotoxicity data are not corroborated by insecticidal activity towards neonate larvae of *S. littoralis*, or the toxic activity against *A. salina*. In the latter tests, the new derivative was nearly inactive, but it showed similar activity in the cytotoxicity assay to kuanoniamine C and D. Thus, it is possible that the different activities observed are not caused by a general cytotoxicity, but may be due to a different mode of action.

ECOLOGY. *Distribution of secondary metabolites in the different sponge parts.* Extraction of the base, fistule and capitum of the sponge yielded significantly different crude extract concentrations. Concentrations increased from the base, with an extract yield of 26.4% of dry mass, 34.2% in the fistule, up to 52.7% crude extract concentration in the capitum (Schupp et al., 1999b).

Concentrations of kuanoniamine C and D increased sharply from the base to the capitum of the sponge. The lowest concentrations of these alkaloids were found in the base, with 0.4% of dry mass for kuanoniamine C and 0.1% of dry mass for kuanoniamine D. The fistule showed a four fold increase of these metabolites, with 1.2% of kuanoniamine C and 0.4% of kuanoniamine D. Highest concentrations were found in the translucent capitum, with 3.5% of dry mass for kuanoniamine C and 1.2% of dry mass for kuanoniamine D, representing a 9 to 12-fold increase in secondary metabolite concentration compared to the base (Schupp et al., 1999b). This huge increase of the two major metabolites kuanoniamine C and D in *Oceanapia* sp. stresses the importance not only to determine an overall yield of secondary metabolites, but also to look at compound concentration at an intraspecimen level. This is important for both ecological investigations and pharmacological screening for new bioactive compounds.

Secondary metabolites are often concentrated in parts where they are first encountered by predators, such as the skin of mollusks or in biologically valuable parts like reproductive regions (Pawlik et al., 1988; Avila & Paul, 1997). When whole organisms are extracted, compounds used by animals to deter possible predators might show lower yields than they actually have in the organs or regions where they are deposited. Therefore, it is possible that bioactive compounds are overlooked in pharmacological screens, because their concentrations are too low.

Fish feeding assays. The methanol fraction was highly deterrent towards reef fishes at base concentration (Table 2). It also deterred feeding by the angelfish *Pomacanthus imperator* in laboratory feeding experiments at the same concentration, although the deterrent effect was not as pronounced as with other reef fishes ($p = 0.047$; $N = 7$; Schupp et al., 1999b).

The field and laboratory fish feeding experiments showed that even the base is chemically well protected. This is surprising, since the base is normally not accessible to fish because it is buried in the sand. A possible explanation could be that the high extract concentrations might be a defense against crabs, flatworms and other sediment-dwelling invertebrates (Schupp et al., 1999b). The laboratory feeding experiments with *P. imperator* demonstrated the potency and broad effectiveness of the compounds, since they deterred the more specialized angelfish in addition to being a deterrent towards generalist reef fishes. Angelfishes are known to feed primarily on ascidians and sponges, and are thought not to be as susceptible to sponge secondary metabolites as are other fishes (Randall & Hartman, 1968; Wulff, 1994).

The field feeding assays with the pure compounds demonstrated that the pyridoacridine alkaloids were at least partly responsible for the deterrent effect of the crude extract. Kuanoniamine C and D clearly deterred fish feeding at fistule concentration (Table 2). Each compound was by itself effective in deterring reef fishes. Schupp et al. (1999b) suggested that it might be important for a sponge to have more then one deterrent chemical, or to increase the overall concentration of defensive compounds, to prevent feeding by different predators. The N-deacyl derivative did not reduce feeding (Table 2). One possible explanation is the low concentration of the compound in the sponge. It was tested at natural concentrations, which is approximately 1/10 of the concentration used for kuanoniamine D.

There are numerous studies showing that benthic invertebrates can reduce predation through the production of secondary metabolites (Bakus et al., 1986; Wylie & Paul, 1989; Paul, 1992; Pawlik, 1993; Ebel et al., 1997; Thacker et al., 1998), but there is little information on the pharmacological activity of these compounds - this study being one of them. One problem might be that researchers looking at the ecological importance of new compounds, seldom perform pharmaceutical screenings, and conversely, those searching for new pharmacologically active substances seldom conduct ecological experiments with the substances or organisms. Fish feeding assays could be used as a first indication for the presence or absence of pharmacologically active substances, and also providing information on the ecological relevance of these compounds as feeding deterrents.

Schupp et al. (1999b) demonstrated that the intraspecimen distribution of secondary metabolites in *Oceanapia* sp. was in accordance with the optimal defense theory (McKey, 1974, 1979; Baldwin & Ohnmeiss 1994). This theory suggests that organisms optimize the production of secondary metabolites and deter possible predators with the least amount of energy, assuming that the production of secondary metabolites is costly. Therefore, biologically important parts (with a high contribution to fitness), like reproductive regions (e.g. the capitum in *Oceanapia* sp.; Hooper et al., 1993), and parts that are first encountered by predators (e.g. the fistule and capitum in *Oceanapia* sp.), should show higher secondary metabolite concentrations. This is certainly the case in *Oceanapia* sp. (Schupp et al., 1999b).

It might be advantageous to keep the optimal defense theory in mind, while collecting and screening for pharmacologically active compounds. By collecting mostly exposed and vulnerable parts of marine invertebrates, it is highly probable that biologically active compounds are present and that they are tested at ecologically relevant concentrations. This could be also interesting from a conservation standpoint, since it should be possible to collect the exposed parts of certain organisms (e.g. sponges), and leave the remainder to regrow. *Oceanapia* sp. can be used again as an example, since it is possible to collect the capitum without severely damaging the sponge. The capitum grows back and can be harvested repeatedly. The harvested amounts are small, but show on the other hand the highest secondary metabolite concentrations.

CONCLUSION

This study demonstrates, that ecological observations and experiments can be useful as initial indicators for possible pharmacological properties of secondary metabolites (Schupp et al., 1999a, 1999b). Field observations and fish feeding experiments demonstrated the biological activity of the pyridoacridine alkaloids found in *Oceanapia* sp. Through different pharmacological screens we were able to show possible physiological targets of the compounds, although these results do not explain the mode of action as a fish deterrent agent.

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